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Impacts of Sample Preparation Methods on Solubility and Antilisterial Characteristics of Essential Oil Components in Milk

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Essential oil components (EOCs) have limited water solubility and are used at much higher concentrations in complex food matrices than in growth media to inhibit pathogens. However, the correlation between solubility and activity has not been studied. The objective of this work was to characterize the solubility of EOCs in solvents and milk and correlate solubility with antilisterial activity. The solubilities of four EOCs, thymol, carvacrol, eugenol, and *trans*-cinnamaldehyde, in water was significantly increased in the presence of 5% (vol/vol) ethanol. In milk, the solubility of EOCs was lower than in water, with lower solubility in higher-fat milk. EOCs applied to milk as stock solutions (in 95% aqueous ethanol) enabled quicker dissolution and higher solubility in milk serum than other methods of mixing, such as end to end, and greater reductions of *Listeria monocytogenes* Scott A after 0 and 24 h. When the EOC concentration detected in milk serum was above the minimum bactericidal concentration, complete inhibition of *L. monocytogenes* in tryptic soy broth resulted. Therefore, the antilisterial properties in milk could be correlated with the solubility by comparison to the minimum inhibitory or bactericidal concentrations of EOCs. While the EOCs applied using ethanol generally had solubility and activity characteristics superior to those of other mixing methods, ethanol is not used to a great extent in nonfermented foods. Therefore, mixing methods without an organic solvent may be more readily adaptable to enhancing the distribution of EOCs in complex food systems.

Plant essential oils (EOs) or EO components (EOCs) are gaining intensive interest as naturally occurring food preservatives due to their broad spectrum of activity against food-borne pathogens and generally recognized as safe (GRAS) regulatory status (1). It is well known that EOs/EOCs perform well in antimicrobial assays conducted using microbial growth media, also called "*in vitro*" tests, but their effectiveness is much reduced in complex food matrices with compounds binding EOs/EOCs. EOs/EOCs are lipophilic and have limited solubility in water (Table 1), mostly below 2 g/liter at around 20°C. The levels of EOCs used in *in vitro* tests, with the MIC and minimum bactericidal concentration (MBC) being the most frequently compared parameters, are usually within the solubility limit (2–4). The level of EOs/EOCs needed to achieve inhibitory and bactericidal activities in foods is highly dependent on composition. Typically, food matrices with hydrophobic food components, like proteins and lipids, can cause significant reductions in antimicrobial activities of EOs/EOCs (5). Despite numerous studies on antimicrobial activities of EOs/EOCs and speculation about food matrix interference (6–8), quantification of the solubility of EOCs in food systems and correlation with antimicrobial activities in food matrices have not been attempted.

In order to improve the distribution of EOs/EOCs in food matrices and reduce interference by food components, various delivery systems, such as emulsions (9, 10) and biopolymer capsules (6, 11–13), have been studied to enhance the antimicrobial activity and reduce the usage level. Commonly, the efficacy of delivery systems is compared to the same concentrations of free (unencapsulated) EOs/EOCs. Tests in food matrices typically require the use of EOs/EOCs above the solubility limit. Various methods have been used to prepare free EOs/EOCs, and thus, comparison of various studies is a challenge. In most studies, organic solvents, such as dimethyl sulfoxide (14), methanol (15, 16), ethylene glycol (17), and ethanol (18–20), are used to prepare stock solutions that are diluted in a test medium to obtain the overall test concentration. The use of water-miscible organic solvents changes the polarity of the aqueous phase when the stock solution is blended with a liquid medium. However, food matrices are typically free of organic solvents. The solubility of EOs/EOCs can also be altered by using surfactants. The polysorbate family of surfactants, including Tween 20 and Tween 80 (21–23), can be used by mixing water, the surfactant, and EOs/EOCs using a vortex mixer. Vortex mixing is a low-shear process that creates an oil-in-water emulsion that is studied as a category of delivery systems, as discussed previously, and changes the distribution properties of EOs/EOCs. Characterization of the solubility characteristics of EOs/EOCs, prepared using various methods in various matrices and correlation of solubility characteristics with antimicrobial activities may thus enable the comparison of studies using free antimicrobials. Such information also provides a rational basis to select free antimicrobials in studying the delivery systems of antimicrobials.

The first objective of the present study was to characterize the solubilities of four commonly studied EOCs, thymol, carvacrol, eugenol, and *trans*-cinnamaldehyde, in various solvents as a result of different preparation methods. The second objective was to compare the antimicrobial activities of EOCs prepared with or without ethanol as a solvent, using *Listeria monocytogenes* Scott A as a model bacterium due to its significance for the microbiological safety of dairy and other food products (24). The third objective was to qualitatively correlate antimicrobial activity with solubility by comparison to the MICs/MBCs of EOCs under the corresponding solvent conditions.

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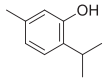
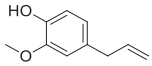
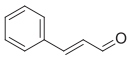
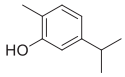
TABLE 1 Water solubility of the studied essential oil components reported in references

Compound	Structure	Solubility in water
Thymol		0.85 g/liter at 25°C (40); 1.25 g/liter at 20°C[a];1.05 g/liter at 20°C (18)
Eugenol		0.64 g/liter at 25°C (41); 1.71 g/liter at 25°C (42)
trans-Cinnamaldehyde		1.1 g/liter at 20°C[a]; 1.76 g/liter at 20°C (18)
Carvacrol		1.25 g/liter at 20°C[a]; 0.83 g/liter at 25°C (40); 0.11 g/liter at 25°C (42); 1.0 g/liter at 37°C (43)

[a] Source, http://www.chemicalbook.com/.

MATERIALS AND METHODS

Materials. Thymol (99% purity) and ethanol (100% and 95%) were from Acros Organics (Morris Plains, NJ). Eugenol (>98% purity), *trans*-cinnamaldehyde (99% purity), and carvacrol (99% purity) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). High-performance liquid chromatography (HPLC) grade water and methanol (>99% purity) were purchased from Fisher Scientific (Pittsburgh, PA). Ultrahigh-temperature-processed skim milk, 2% reduced-fat milk, and full (3.3%)-fat milk (Simple Truth Organic, San Diego, CA) were purchased from a local grocery store.

Solubility of EOCs in water, TSB, 2% (vol/vol) ethanol, and 5% (vol/vol) ethanol. The solvents studied were water and low concentrations (2 and 5% [vol/vol]) of aqueous ethanol. To determine the solubilities of EOCs in different solvents, 5 g/liter of an EOC was mixed with each solvent and stirred using a stir plate (Fisher Scientific) at room temperature (21°C) for at least 24 h to reach solubilization equilibrium. The mixture was filtered through a 0.45-μm polyvinylidene difluoride (PVDF) membrane (Fisher Scientific), and 1 ml of the permeate was used for HPLC analysis (see below). The solubility in tryptic soy broth (TSB) was tested similarly. Each sample was analyzed in triplicate.

Solubility of EOCs in milk prepared by different methods. Three methods were compared for mixing EOCs with milk at room temperature (21°C). In the end-to-end shaker method (ETEM), an EOC was weighed directly into glass tubes according to the mass expected from the overall concentration (5 g/liter for thymol and carvacrol, 7 g/liter for eugenol, and 9 g/liter for *trans*-cinnamaldehyde), 9 ml milk plus 1 ml water was added, and the tubes were capped and attached to an end-to-end shaker (Thermo Scientific, Hudson, NH) for 0, 5, 15, and 30 min or 24 h. In the vortex method (VM), the capped tubes with milk and EOC were agitated with a vortex mixer at low speed for different durations than for the ETEM. In the ethanol predissolving method (EPM), stock solutions of EOCs were prepared in 10-ml volumetric flasks at a concentration of 100 g/liter for thymol and carvacrol, 140 g/liter for eugenol, and 180 g/liter for *trans*-cinnamaldehyde in 95% aqueous ethanol. Then, 500 μl of the stock solution was added to 8.5 ml milk with 1 ml water and mixed with the vortex mixer for ~10 s to obtain the same EOC final concentrations as in the ETEM and VM. Samples were assayed shortly after mixing (0 h) and after 24 h.

The above-mentioned milk samples were acidified to pH 4.6 to precipitate caseins. After centrifugation at 4,564 × *g* for 5 min (Sorvall RC-5B Plus; Sorvall, Newtown, CT) at 20°C, the supernatant was filtered through a 0.45-μm PVDF membrane (Fisher Scientific) to obtain the permeate for HPLC analysis. The permeate as prepared is referred to as milk serum here.

HPLC apparatus and quantification conditions. A reversed-phase HPLC system (1200 series; Agilent Technologies, Waldbronn, Germany) was used to quantify EOCs. The system consisted of a quaternary pump module, a degasser, an autosampler, a temperature-controlled column chamber, and an Agilent diode array and multiple-wavelength detector. Chromatograms were recorded and integrated by the 1200 LC Chromatography Data System. All experiments were performed on an Agilent Zorbax Eclipse Plus C_{18} HPLC column (5 μm; 150 mm by 4.6 mm; Agilent, Palo Alto, CA) protected by a Zorbax Eclipse Plus C_{18} guard column (4.6 by 12.5 mm; 5 μm). The sample injection volume was 10 μl, and the detection wavelength was 274 nm. A binary solvent mixture of water (solvent A) and methanol (solvent B) in different proportions was used as the mobile phase. The optimized elution conditions were a linear gradient from 20% B to 80% B within 20 min, an isocratic step with 80% B for 5 min, and a linear gradient from 80% B to 20% B in 5 min. The flow rate was 0.5 ml/min, and the column chamber was controlled at 25°C.

External standards were used to establish calibration curves. EOCs were dissolved in methanol at 2 g/liter in 10-ml volumetric flasks to become transparent solutions and were filtered through the 0.45-μm PVDF membrane before dilution in methanol to the concentration range shown in Table 2. At least five concentrations within the range were used to generate a calibration curve correlating the peak area (A) and the EOC concentration (C). To test the recovery of EOCs in milk serum samples, a known amount of each EOC was spiked in the serum prepared as described above from full-fat milk without EOC at an overall concentration of 0.05, 0.1, and 0.2 g/liter for thymol, eugenol, and carvacrol and 0.005, 0.01, and 0.02 g/liter for *trans*-cinnamaldehyde. The samples were then filtered as described above for quantification using HPLC. The recovery percentage was calculated based on the amount of EOC estimated using the calibration curve with respect to the amount of EOC spiked in the milk serum.

Culture preparation. The *L. monocytogenes* strain Scott A culture was obtained from the culture collection of the Department of Food Science and Technology at the University of Tennessee (Knoxville, TN). The stock culture, stored in glycerol at −20°C, was transferred into TSB at 32°C for 2 consecutive days. *L. monocytogenes* was grown for 18 h at 32°C before dilution to ~7.0 log CFU/ml in TSB as the working culture.

Microplate dilution assay to determine MICs and MBCs of EOCs against *L. monocytogenes*. A broth dilution method with modification was used to determine the MICs and MBCs of EOCs against *L. monocytogenes* (25, 26). Each EOC was prepared at a stock concentration of 100 g/liter in 95% ethanol in 10-ml volumetric flasks. The working solution with 4 g/liter of each EOC was prepared by diluting the 100-g/liter stock solution in TSB. The 4-g/liter working solution was further diluted in TSB to an EOC concentration of 0.1 to 4 g/liter, with 0.2-g/liter increments. To study the impacts of ethanol, the 4-g/liter working solution was diluted from the 100-g/liter stock solution with ethanol and TSB to an ethanol

TABLE 2 Characteristics of HPLC calibration curves and the measured solubility of essential oil components in water, TSB, 2% (vol/vol) ethanol, and 5% (vol/vol) ethanol at 21°C[a]

Compound	Peak area-concn calibration curve			Recovery (%)	Solubility in[b]:			
	Regression equation	R^2	Concn range (mg/ml)		Water (g/liter)	TSB (g/liter)	Ethanol (vol/vol) 2%	Ethanol (vol/vol) 5%
Thymol	$A = 14{,}558\ C$	0.9998	0–1	96.56 ± 1.43	0.48 ± 0.02 b	0.43 ± 0.01 b	0.52 ± 0.02 ab	0.61 ± 0.05 a
Carvacrol	$A = 13{,}789\ C$	0.9999	0–1	99.64 ± 1.84	0.45 ± 0.02 b	0.41 ± 0.00 b	0.46 ± 0.00 b	0.57 ± 0.02 a
Eugenol	$A = 14{,}190\ C$	0.9997	0–1	96.37 ± 2.74	1.35 ± 0.1 b	1.42 ± 0.01 b	1.49 ± 0.02 b	1.63 ± 0.12 a
trans-Cinnamaldehyde	$A = 134{,}163\ C$	0.9992	0–0.1	96.70 ± 3.83	1.41 ± 0.05 bc	1.51 ± 0.03 c	1.63 ± 0.04 ab	1.72 ± 0.13 a

[a] Numbers are mean ± standard error ($n = 3$).
[b] Different lowercase letters next to the numbers in the same row represent significant differences ($P < 0.05$).

concentration of 5% (vol/vol) or 10% (vol/vol). TSB was mixed with ethanol to an ethanol concentration of 5% (vol/vol) or 10% (vol/vol), which was then used to dilute the 4-g/liter EOC working solution to obtain 0.1 to 4 g/liter EOC in 5% (vol/vol) or 10% (vol/vol) aqueous ethanol. The final EOC solution was added at 120 μl in each well of the sterile 96-well plate. A total of 120 μl of the working culture with 6 log CFU/ml *L. monocytogenes* was added to each well, corresponding to overall EOC concentrations of 0.05 to 2 g/liter. The plates were incubated at 32°C or 21°C for 24 h. The MIC was the lowest concentration that did not show any visible growth (25, 26). Using the results of the MIC assay, 20 μl of each culture broth from the wells with EOC concentrations equal to or higher than the MIC was transferred onto tryptic soy agar (TSA) plates and incubated for another 48 h at 32°C or 21°C. The lowest concentration of each EOC corresponding to the absence of *L. monocytogenes* growth on TSA was treated as the MBC. Fresh working cultures were grown separately in triplicate, and each EOC concentration was tested four times for each working culture.

Antilisterial activity of EOCs in milk. The ETEM and EPM were chosen to compare differences of antilisterial properties in milk as affected by the preparation method. *L. monocytogenes* working culture (1 ml), prepared to ~7.0 log CFU/ml in TSB as described above, was added to milk to obtain an overall population of around 6 log CFU/ml. In the ETEM, 9 ml milk was mixed with 1 ml of the working culture, while 8.5 ml milk was mixed with 1 ml of the working culture and 0.5 ml of the EOC stock solution (in 95%) in the EPM. The negative control was a mixture of milk and working culture, while the ethanol control had an additional 500 μl of 95% or 70% ethanol to test the growth of *L. monocytogenes* in a low overall concentration of ethanol (~4.75% [vol/vol] and ~3.5% [vol/vol]). *L. monocytogenes* was enumerated by the pour plate method on TSA plates before (treated as the 0-h time point) and after 24 h of incubation at room temperature (21°C), and the log reduction was determined. For the 0-h time point, the working culture was mixed into milk by vortex mixing as the last step, and the plating on TSA was conducted immediately afterward. Each treatment was repeated in three independent replicates, with fresh working culture grown separately in each replication ($n = 3$). The qualitative correlation of antilisterial activity and EOC concentration was done by comparing the EOC concentration in milk serum to the MIC/MBC of EOCs under the corresponding solvent conditions.

Statistical analysis. All experiments were conducted in triplicate, and the data were expressed as the mean ± standard error. Statistical significance (analysis of variance [ANOVA]) was determined using SAS software (version 9.3; SAS Institute, Cary, NC). Differences between pairs of means were analyzed using a *post hoc* Tukey test at a significance level of 0.05.

RESULTS

Validation of the HPLC assay for quantifying EOC solubility in solvents and milk. The calibration curves of the HPLC assay for the four EOCs are presented in Table 2. The linear range for thymol, carvacrol, and eugenol was from 0 to 1 g/liter, while the upper limit of *trans*-cinnamaldehyde was 0.1 g/liter. The R^2 values of all calibration curves were greater than 0.999. The chromatograph of the serum from full-fat milk did not show milk component peaks appearing at the elution time range (19 to 26 min) of EOCs (chromatographs not shown). The recovery percentage was higher than 95% for all the samples tested, indicating the applicability of the calibration curves to the samples prepared from milk. Therefore, the established HPLC conditions can be used to quantify the solubility of EOCs in both solvents and milk.

Solubility of EOCs in water, TSB, 2% (vol/vol) ethanol, and 5% (vol/vol) ethanol. The solubilities of thymol, carvacrol, eugenol, and *trans*-cinnamaldehyde in water at room temperature (~21°C) were 0.48, 0.45, 1.35, and 1.41 g/liter, respectively (Table 2). The solubility data tested under the studied conditions show some differences from the literature, which also varied significantly (Table 1). Generally, the solubilities of these four EOCs were not different in water and TSB ($P > 0.05$) and increased with the presence of low levels of ethanol, with those in the 5% ethanol treatment being significantly (~20 to 30%) higher than those in water.

Amounts of EOCs dissolved in milk using different mixing methods. The ETEM, VM, and EPM using 95% ethanol were first compared for the thymol concentration in the serum of full-fat milk. Mixing 5 g/liter of thymol with milk using the ETEM and VM for up to 30 min resulted in significant increases in the thymol concentration, while the increase was insignificant ($P > 0.05$) after mixing for 24 h (Fig. 1). Less than 0.15 g/liter thymol was detected in the serum and was not different ($P > 0.05$) between the two agitation methods. The thymol concentrations in milk serum using the EPM were similar after different mixing durations (0.18 g/liter at 0 h versus 0.17 g/liter at 24 h) but were higher ($P < 0.05$) than that using the ETEM and VM. The 20% greater thymol concentration in the serum prepared using the EPM compared to that using the ETEM/VM is similar to the results with water and 5% ethanol (Table 2). Even though a low speed was used, VM caused visible structural changes in the milk after long mixing times (Fig. 2), which contrasted with no visible changes in the milk using the ETEM. Additionally, the shear force of VM can create emulsions of EOs/EOCs if samples contain emulsifiers (e.g., surface-active proteins in milk), which would not be characteristic of "free" EOCs. Therefore, the ETEM, which is easier to use to reach the solubility (in 30 min) than the VM, is recommended to maintain the solubility properties of EOCs.

The four EOCs were then studied in the skim, 2% fat, and 3.3% fat milk products prepared by mixing for 30 min using the ETEM and short-time vortexing using the EPM. The concentration of EOCs in the milk serum was determined after 0 and 24 h (Fig. 3)

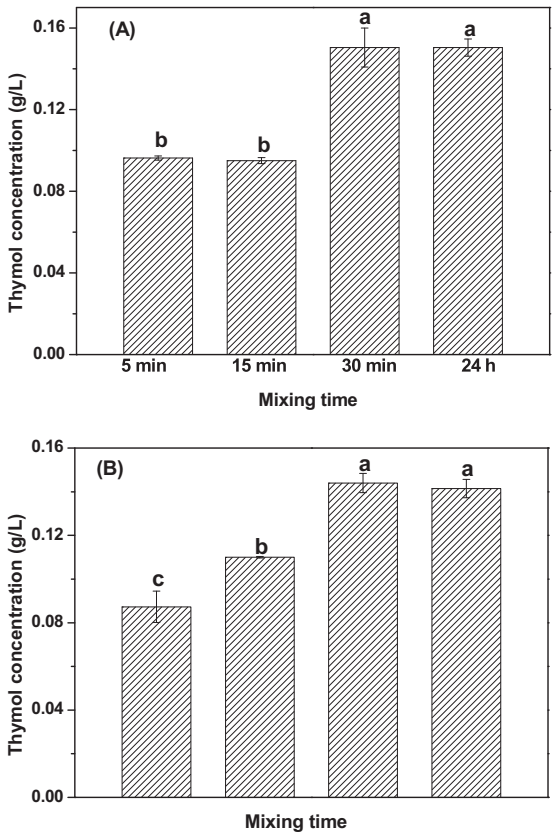


FIG 1 Thymol concentrations detected in serum of full-fat milk after mixing with an end-to-end shaker (A) or vortexing (B) for different durations. The error bars indicate standard errors ($n = 3$). Different letters above the bars indicate differences between mean values ($P < 0.05$).

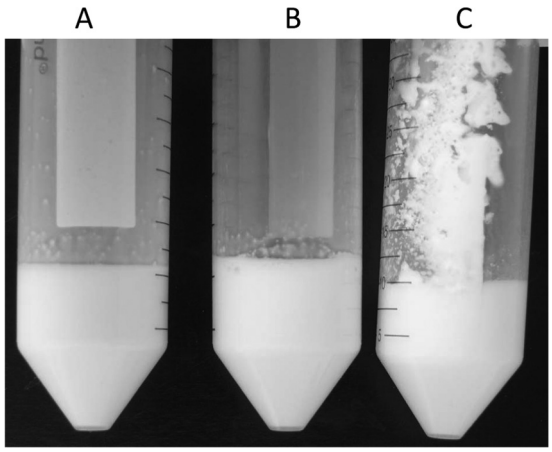


FIG 2 Appearance of full-fat milk mixed with 5 g/liter thymol crystals using an end-to-end shaker for 24 h (A), 500 μl of a stock solution with 100 g/liter thymol in 95% aqueous ethanol (B), and 5 g/liter thymol crystals after low-speed vortexing for 24 h (C).

and was found to be affected by both the mixing method (ETEM or EPM) and the milk fat composition. The EOC concentration in the milk serum decreased with an increase in fat content ($P < 0.05$). The ETEM duration did not show any significant effects on carvacrol and eugenol solubility (Fig. 3B and C), but the concentration of *trans*-cinnamaldehyde in skim milk decreased after 24 h (Fig. 3D). The EOC concentration in the milk serum prepared by the EPM was consistently higher than that with the corresponding ETEM treatment, and the effect of storage time was insignificant (Fig. 3).

MICs and MBCs of EOCs in TSB and correlation with solubility. The MICs and MBCs of EOCs in TSB with different amounts of ethanol at 32°C and 21°C are listed in Table 3. For ethanol alone, the MICs were 10% (vol/vol) and 5% (vol/vol) at 32°C and 21°C, respectively, while the MBC was 20% (vol/vol) at both temperatures. The MICs of thymol and carvacrol were 0.2 g/liter at both 32 and 21°C, except the 5% ethanol treatment at 21°C, which had a MIC below the lowest EOC concentration tested due to sufficient inhibition (MIC) by 5% ethanol alone. The MBC of thymol and carvacrol was 0.3 g/liter under all tested conditions. Conversely, the combination of ethanol with eugenol and *trans*-cinnamaldehyde showed enhanced antilisterial properties. With the exception of the MIC of *trans*-cinnamaldehyde (0.2 g/liter) at 32°C, reduced MICs and MBCs were observed at a higher level of ethanol. Overall, the MBCs of the four EOCs did not differ between 32°C and 21°C, but the MIC was lower at 21°C for *trans*-cinnamaldehyde and eugenol.

Increased solubility of EOCs in the presence of low concentrations of ethanol had different effects on the MIC and MBC depending on the type of EOC. For thymol and carvacrol, the MBCs (0.3 g/liter) (Table 3) at 21°C were 69.8% and 73.2% of the solubility in TSB (Table 2), respectively. Because the MBC is well below the solubility, the same MIC and MBC at the three ethanol concentrations tested were observed (Table 3). Conversely, the MBCs of eugenol and *trans*-cinnamaldehyde with the lowest ethanol concentration (Table 3) at 21°C were 91.5% and 92.7% of their solubility in TSB (Table 2), respectively, and the increased solubility by low concentrations of ethanol reduced the MIC and MBC.

Antilisterial activities of EOCs in milk and correlation with solubility. The log reductions of *L. monocytogenes* in the three milk samples after treatment by EOCs using the ETEM and EPM are presented in Tables 4 to 6. When 500 μl of 70% and 90% aqueous ethanol was mixed with 8.5 ml milk and 1 ml culture, the overall concentrations of ethanol corresponded to around 3.5% (vol/vol) and 4.75% (vol/vol), respectively. Growth of *L. monocytogenes* was observed in both 3.5% (vol/vol) and 4.75% (vol/vol) ethanol controls but was slightly lower than that of the negative control ($P < 0.05$). As 5% (vol/vol) ethanol was near the MIC at 21°C (Table 3), this agrees with the slight inhibition of growth of the microorganism (Tables 4 to 6).

In skim milk (Table 4), 5 g/liter thymol and carvacrol inactivated *L. monocytogenes* to below the detection limit with the ETEM or EPM, and no recovery was observed after 24 h. The thymol concentrations in the aqueous phase (Fig. 3) at 0 h and 24 h were both higher than the MBC, 0.3 g/liter (Table 3). For the 2.5-g/liter thymol treatment, the difference between the ETEM and EPM was apparent shortly after mixing, with only a 0.3 log CFU/ml reduction for the former and below the detection limit for the latter. For the 2.5-g/liter carvacrol treatment in skim milk, *L. monocytogenes* was not detected in any treatment.

For *trans*-cinnamaldehyde at 4.5 or 9 g/liter in skim milk (Table 4), a ca. 1 log CFU/ml reduction occurred after 24 h in the ETEM compared with a >5 log CFU/ml reduction for the EPM. The differences between the ETEM and the EPM were also ob-

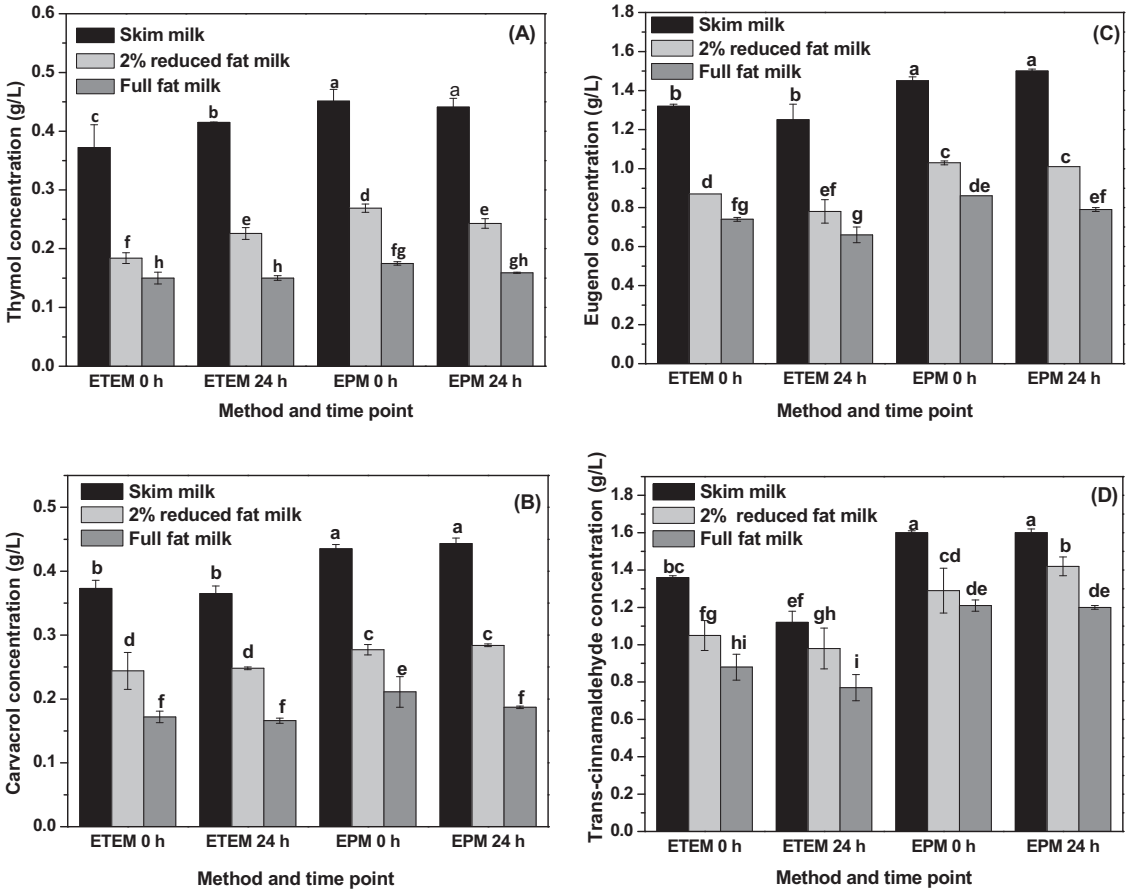


FIG 3 Concentrations of EOCs in sera of milk with different fat levels after mixing with 5 g/liter thymol (A), 5 g/liter carvacrol (B), 7 g/liter eugenol (C), and 9 g/liter *trans*-cinnamaldehyde (D) using an end-to-end shaker for 30 min (ETEM) or with a stock solution in 95% ethanol (EPM) before (0 h) and after incubation at 21°C for 24 h. The error bars indicate standard errors ($n = 3$). Different letters above the bars indicate differences between mean values ($P < 0.05$).

TABLE 3 MICs and MBCs of the four EOCs against *L. monocytogenes* Scott A in different concentrations of ethanol at 32°C and 21°C determined using the broth dilution method ($n = 4$)

Compound	Ethanol concn (%)	MIC (g/liter)[b]		MBC (g/liter)[b]	
		32°C	21°C	32°C	21°C
Thymol	<1.3[a]	0.2	0.2	0.3	0.3
	2.5	0.2	0.2	0.3	0.3
	5	0.2	<0.05[c]	0.3	0.3
Carvacrol	<1.3[a]	0.2	0.2	0.3	0.3
	2.5	0.2	0.2	0.3	0.3
	5	0.2	<0.05[c]	0.3	0.3
Eugenol	<1.3[a]	0.8	0.5	1.3	1.3
	2.5	0.7	0.4	1.1	1.1
	5	0.5	<0.2[c]	1.0	1.0
trans-Cinnamaldehyde	<1.3[a]	0.2	0.2	1.4	1.4
	2.5	0.2	0.1	1.2	1.2
	5	0.2	<0.05[c]	0.9	0.9
Ethanol		10%	5%	20%	20%

[a] The concentration derived from the stock solution with 95% aqueous ethanol to prepare an overall EOC concentration of 1.4 g/liter.
[b] MIC is in g/liter for all compounds except ethanol.
[c] Below the lowest concentration used in the assay.

served for eugenol treatments at 7 and 3.5 g/liter. With 7 g/liter eugenol, *L. monocytogenes* was initially reduced to below the detection limit. Recovery was observed for the ETEM treatment after 24 h, but not the EPM treatment. For the ETEM, 3.5 g/liter eugenol did not cause any apparent reduction of *L. monocytogenes* initially and only a 2.8 log CFU/ml reduction after 24 h. The 3.5-g/liter eugenol treatment prepared with the EPM corresponded to 4.7 log CFU/ml reduction after 0 h, and *L. monocytogenes* was completely inactivated after 24 h. The differences were in agreement with the solubility characteristics shown in Fig. 3C, i.e., a reduction of the eugenol concentration after 24 h for the ETEM (although not statistically significant) compared to a slight increase in the EPM. For *trans*-cinnamaldehyde, the viable *L. monocytogenes* population was not below the detection limit. As shown in Fig. 3D, the detected concentration of *trans*-cinnamaldehyde in the milk serum was around 1.4 g/liter at time zero and 1.2 g/liter at 24 h for the ETEM, both of which were equal to or less than the MBC at <1.3% ethanol (1.4 g/liter) (Table 3). Conversely, the *trans*-cinnamaldehyde concentration in the milk serum was about 1.6 g/liter in the EPM at both 0 and 24 h (Fig. 3D), which was higher than the MBC (0.9 g/liter) (Table 3), resulting in complete inactivation.

In 2% reduced-fat milk (Table 5), the inactivation of *L. monocytogenes* was reduced compared to skim milk at the same concen-

TABLE 4 Log reduction of *L. monocytogenes* Scott A treated with EOCs mixed via an end-to-end shaker or an ethanol stock solution in skim milk at 21°C

Compound	Overall concn	Log reduction[a]			
		End-to-end shaker		95% (vol/vol) ethanol	
		0 h	24 h	0 h	24 h
Thymol	5 g/liter	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a
	2.5 g/liter	0.3 ± 0.5 b	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a
Carvacrol	5 g/liter	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a
	2.5 g/liter	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a	>5.3 ± 0.2 a
trans-Cinnamaldehyde	9 g/liter	0.2 ± 0.3 c	1.3 ± 0.5 b	0.4 ± 0.5 c	>5.3 ± 0.2 a
	4.5 g/liter	0.1 ± 0.2 c	0.8 ± 0.2 b	0.1 ± 0.4 b	5.0 ± 0.7 a
Eugenol	7 g/liter	>5.3 ± 0.2 a	4.2 ± 0.8 b	>5.3 ± 0.2 a	>5.3 ± 0.2 a
	3.5 g/liter	0.3 ± 0.3 c	2.8 ± 0.4 b	4.7 ± 1.3 a	>5.3 ± 0.2 a
Controls					
Negative control (no antimicrobial)			−2.1 ± 0.1 C		
Ethanol	4.75% (vol/vol)	0.1 ± 0.2 b	−1.2 ± 0.1 a A		
	3.5% (vol/vol)	0.1 ± 0.2 b	−1.6 ± 0.2 a B		

[a] The log reduction is the difference between the population of *L. monocytogenes* in the negative control at time zero and that after mixing with EOCs using an end-to-end shaker for 30 min or a stock solution in 95% ethanol using brief vortexing before (0 h) or after incubation at 21°C for 24 h. The population of *L. monocytogenes* in the negative control at 0 h was 6.2 ± 0.5 log CFU/ml. The numbers are means ± standard errors ($n = 3$). The detection limit was 1 log CFU/ml. Different lowercase letters next to the numbers in the same row and uppercase letters next to the controls in the same column represent significant differences ($P < 0.05$).

trations of EOCs (Table 4). For each EOC, even though treatments with the lower concentrations showed no or insignificant (except for 4.5 g/liter *trans*-cinnamaldehyde) inactivation of *L. monocytogenes* after 24 h, there was still inhibition compared to the control of *L. monocytogenes* in EOC treatments prepared with the EPM. At higher EOC concentrations, the ETEM treatments with 5 g/liter thymol and 5 g/liter carvacrol reduced *L. monocytogenes* by ~1.5 log CFU/ml after 24 h, which was lower than the EPM, which showed complete inactivation (>5.3 CFU/ml) after 24 h ($P < 0.05$). The solubility of thymol and carvacrol in 2% reduced-fat milk was 0.18 g/liter and 0.2 g/liter when prepared with the ETEM (Fig. 3), both of which are around the MIC but lower than the MBC (Table 3). When thymol and carvacrol were dissolved via the EPM, the solubilities of these two EOCs were around 0.25 g/liter (Fig. 3). Even though this was slightly lower than the MBC (0.3 g/liter), *L. monocytogenes* was inactivated to below the detection

TABLE 5 Log reduction of *L. monocytogenes* Scott A treated with EOCs mixed via an end-to-end shaker or an ethanol stock solution in 2% reduced-fat milk at 21°C

Compound	Overall concn	Log reduction[a]			
		End-to-end shaker		95% (vol/vol) ethanol	
		0 h	24 h	0 h	24 h
Thymol	5 g/liter	0.1 ± 0.2 c	1.3 ± 0.8 b	0.5 ± 0.4 c	>5.3 ± 0.1 a
	2.5 g/liter	0.1 ± 0.2 a	−1.5 ± 0.5 b	0.1 ± 0.2 a	−0.9 ± 0.2 b
Carvacrol	5 g/liter	0.3 ± 0.2 b	1.8 ± 1.6 b	0.6 ± 0.1 b	>5.3 ± 0.1 a
	2.5 g/liter	0.0 ± 0.2 a	−1.2 ± 0.3 b	0.3 ± 0.2 a	−0.8 ± 0.2 b
trans-Cinnamaldehyde	9 g/liter	0.3 ± 0.2 c	1.0 ± 0.4 b	0.3 ± 0.3 c	>5.3 ± 0.1 a
	4.5 g/liter	0.2 ± 0.2 a	0.7 ± 0.3 a	0.2 ± 0.3 a	1.4 ± 1.4 a
Eugenol	7 g/liter	0.2 ± 0.2 b	0.5 ± 0.4 b	0.3 ± 0.5 b	3.4 ± 1.4 a
	3.5 g/liter	0.2 ± 0.2 a	−1.7 ± 0.1 b	0.2 ± 0.4 a	−0.7 ± 0.9 ab
Controls					
Negative control (no antimicrobial)		0	−2.2 ± 0.1 C		
Ethanol	4.75% (vol/vol)	0.0 ± 0.1 b	−1.1 ± 0.1 a A		
	3.5% (vol/vol)	0.0 ± 0.1 b	−1.7 ± 0.2 a B		

[a] The log reduction is the difference between the population of *L. monocytogenes* in the negative control at time zero and that after mixing with EOCs using an end-to-end shaker for 30 min or a stock solution in 95% ethanol using brief vortexing, before (0 h) or after incubation at 21°C for 24 h. The population of *L. monocytogenes* in the negative controls at 0 h was 6.3 ± 0.1 log CFU/ml. The numbers are means ± standard errors ($n = 3$). The detection limit was 1 log CFU/ml. Different lowercase letters next to the numbers in the same row and uppercase letters next to the controls in the same column represent significant differences ($P < 0.05$).

TABLE 6 Log reductions of *L. monocytogenes* Scott A treated with EOC mixed via an end-to-end shaker and ethanol stock solution in full-fat milk at 21°C

Compound	Overall concn	Log reduction[a]			
		End-to-end shaker		95% (vol/vol) ethanol	
		0 h	24 h	0 h	24 h
Thymol	5 g/liter	−0.1 ± 0.2 ab	−0.8 ± 0.1 b	0.0 ± 0.0 a	0.3 ± 0.5 a
	2.5 g/liter	−0.1 ± 0.1 a	−1.9 ± 0.1 b	0.0 ± 0.2 a	−1.2 ± 0.1 b
Carvacrol	5 g/liter	−0.1 ± 0.1 b	−0.2 ± 0.3 b	0.4 ± 0.1 a	0.5 ± 0.6 a
	2.5 g/liter	−0.1 ± 0.0 a	−2.0 ± 0.0 c	0.0 ± 0.1 a	−1.0 ± 0.1 b
trans-Cinnamaldehyde	9 g/liter	0.1 ± 0.1 b	0.8 ± 0.3 a	0.1 ± 0.2 b	1.3 ± 0.9 a
	4.5 g/liter	0.0 ± 0.1 a	0.3 ± 0.3 a	0.1 ± 0.3 a	0.3 ± 0.2 a
Eugenol	7 g/liter	0.0 ± 0.1 a	−1.2 ± 0.2 b	0.1 ± 0.2 a	0.4 ± 0.2 a
	3.5 g/liter	0.0 ± 0.0 a	−1.8 ± 0.2 c	0.1 ± 0.2 a	−1.0 ± 0.3 b
Controls					
Negative control (no antimicrobial)			−2.2 ± 0.1 C		
Ethanol	4.75% (vol/vol)	0.1 ± 0.2 b	−1.0 ± 0.2 a A		
	3.5% (vol/vol)	0.1 ± 0.1 b	−1.6 ± 0.2 a B		

[a] The log reduction is the difference between the population of *L. monocytogenes* in the negative control and that after mixing with EOCs using an end-to-end shaker for 30 min or a stock solution in 95% ethanol using brief vortexing, before (0 h) or after incubation at 21°C for 24 h. The population of *L. monocytogenes* at time 0 was 6.4 ± 0.1 log CFU/ml. Different lowercase letters next to the numbers in the same row and uppercase letters next to the controls in the same column represent significant differences ($P < 0.05$). The numbers are means ± standard errors ($n = 3$).

limit. For eugenol, the solubilities in both the ETEM and EPM (Fig. 3C) were lower than the MBC (Table 3), but the higher solubility in the EPM resulted in better log reduction at 24 h ($P < 0.05$). *trans*-Cinnamaldehyde in 2% reduced-fat milk showed a trend similar to that in skim milk. *trans*-Cinnamaldehyde (9-g/liter) prepared with the EPM reduced *L. monocytogenes* to below the detection limit after 24 h, while the same concentration in the ETEM caused only a 1-log-unit reduction of *L. monocytogenes*. As shown in Fig. 3D, the *trans*-cinnamaldehyde concentration in the serum of 2% reduced fat milk was higher than the MBC (0.9 g/liter) (Table 3) at 0 h and 24 h when prepared by the EPM. In contrast, the concentration of *trans*-cinnamaldehyde in the serum of 2% reduced-fat milk (~1 g/liter) (Fig. 3D) was lower than the MBC (1.4 g/liter) (Table 3) but higher than the MIC (0.2 g/liter) when prepared by the ETEM, which agrees with ca. 1 log CFU/ml reduction at both antimicrobial concentrations (Table 5).

Table 6 shows the antilisterial activities of EOCs applied in full-fat milk. For 5 g/liter thymol and carvacrol, growth of *L. monocytogenes* (negative log reductions) was observed in the ETEM treatments, while no growth (bacteriostasis) was seen with the EPM treatment. This again agrees with the EOC concentrations in the milk serum, i.e., much lower than 0.2 g/liter (MIC) in the ETEM and near 0.2 g/liter in the EPM (Fig. 3A and B). Conversely, it was observed that 7 g/liter eugenol inactivated *L. monocytogenes* by 0.4 log CFU/ml after 24 h when prepared with the EPM compared to the >1 log CFU/ml growth for the ETEM. The results corresponded to the concentration of eugenol in the milk serum (<0.8 g/liter) (Fig. 3C), being lower than the MBC (1.3 g/liter) when prepared with the ETEM. Conversely, the concentration of eugenol in the serum of full-fat milk via the EPM was around 0.9 g/liter, which was close to the MBC of eugenol in TSB with 5% ethanol (1.0 g/liter) (Table 3). For *trans*-cinnamaldehyde, the concentration in the serum of full-fat milk via the ETEM was lower than its MBC (1.4 g/liter) (Table 3) but much higher than its MIC (0.2 g/liter) (Table 3) and corresponded to 0.8 and 1.3 log CFU/ml reductions in the ETEM and the EPM, respectively. The difference in log reductions between the ETEM and the EPM for *trans*-cinnamaldehyde was not significant. It was also observed that, while the concentration of *trans*-cinnamaldehyde dissolved via the EPM (Fig. 3D) was higher than the MBC, complete inactivation of *L. monocytogenes* was not seen.

DISCUSSION

The MICs and MBCs in the present study generally agree with the literature. The MICs and MBCs of thymol and eugenol obtained at 32°C in TSB were similar to those in our earlier study obtained using a 2-fold serial dilution method (27), where the MICs and MBCs of thymol and eugenol were 0.187 g/liter and 0.375 g/liter, and 0.75 g/liter and 1.5 g/liter, respectively. Similar MICs of thymol and carvacrol against *Listeria innocua* were observed by other researchers (28). The MIC and MBC of *trans*-cinnamaldehyde against *L. innocua* at 35°C were reported to be 0.5 g/liter and 2 g/liter, respectively (29), which are similar to the results in the present study (Table 3). The MIC of ethanol obtained in the present study agrees with a previous report showing strong inhibition of *L. monocytogenes* in TSB yeast extract by 5% (vol/vol) ethanol at 35°C (30).

The reduced activity of EOCs in complex food systems (milk in the present study) compared to that in microbial growth media and simple food systems also agrees with earlier studies. For example, 0.5 g/liter thymol was needed in apple cider to inhibit the growth of *Escherichia coli* O157:H7 ATCC 43889 and ATCC 43894 and *L. monocytogenes* strains Scott A and 101, while 9 times this concentration was needed for similar inhibition in 2% reduced-fat milk (6). In milk with various levels of milk fat, the MICs of eugenol against *E. coli* O157:H7 were 3.5, 4.5, and 5.5 g/liter in skim, 2% reduced-fat, and full-fat milk, respectively, and the MBCs were 1 g/liter higher than the MICs in the same media (6). For *L.*

monocytogenes Scott A and 101, the MICs of eugenol were 3.5 g/liter in skim milk, 4.5 g/liter in 2% reduced-fat milk, and 6.5 g/liter in full-fat milk, while the MBCs were all 6.5 g/liter (11). Carvacrol reduced the growth of *Bacillus cereus* in brain heart infusion broth at 0.06 g/liter, but a 50-fold-higher concentration was needed to achieve the same effect in various soups (7). A 100-fold increase in the concentration of a mixture of EO extracts from rosemary, sage, and citrus in glycerol was needed to achieve similar inhibition of *L. monocytogenes* in soft cheese compared to treatments in TSB (8).

Proteins and lipids have been proposed as components interfering with antimicrobial activities of EOCs in food matrices (5). Milk is a good model food system to study such interference because it contains both proteins and lipids, and milk products with various fat levels and consistent protein content are readily available. Although the decreased antimicrobial activity of EOCs in milk with a higher fat content has been frequently observed (6, 11, 27, 31), the physicochemical mechanisms remain unclear. Bacteria have hydrophilic surfaces and are expected to be present in the aqueous phase (31, 32). Physically, the hydrophobic nature of EOCs enables their dissolution in phospholipids of the bacterial cell membrane, and a sufficient quantity of EOCs in phospholipids is needed to cause substantial changes in membrane structures and cell metabolism to inhibit and inactivate bacteria (1, 33). Because the surfaces of bacterial cells have well-organized structures, it is likely that only the dissolved EOC molecules have small enough dimensions to diffuse through the surface structure to access phospholipids. In microbial growth media, the MICs and MBCs (Table 3) of EOCs are typically below their solubility (Table 2), and all EOC molecules are available for interacting with bacteria. In milk, a much higher concentration of EOCs is needed to inhibit the growth of bacteria. When EOCs are added to milk above the solubility limit, a fraction of EOCs is expected to be dissolved and the remainder is present as dispersed particles. The dissolved EOCs can diffuse into porous casein micelles (34); bind with whey proteins, such as β-lactoglobulin, known for its hydrophobic barrel available for loading hydrophobic compounds (35); or be attracted by fat globules. Casein micelles and whey proteins are present at about 2.6% (wt/wt) and 0.63% (wt/wt) in milk (36) and cause about 10% reduction in the dissolved EOCs (Fig. 3, skim milk samples, versus Table 2). Fat globules appear to have strong ability to bind EOCs, causing a much lower concentration of EOCs in the milk serum than the solubility in simple solvents (Fig. 3 and Table 2). The initially undissolved EOCs in the form of dispersed particles can continue to be dissolved in the continuous phase when there is a concentration gradient across the particle surface, and this portion of the dissolved EOCs also can bind with dairy proteins and fat globules. The dynamics of dissolving and binding processes determine the amount of EOCs dissolved in the milk serum that becomes available to interact with bacteria. This enables the correlation of the EOC concentration in the milk serum and antilisterial properties by comparison to the MIC and MBC data obtained in TSB, as presented above.

Ethanol is routinely used as a solvent to prepare stock solutions of EOCs before dilution to the required concentrations for microbiological experiments. Ethanol is considered bactericidal at high concentrations (60% to 75%) (37), and the impacts of low concentrations of ethanol on antimicrobial activities of EOCs are usually not addressed. The presence of low concentrations of ethanol lowers the polarity of the continuous phase and therefore increases the solubility (Table 2) and EOC concentration in the milk serum (Fig. 3). This impacts the MICs and MBCs for EOCs with solubility comparable to the MBC (e.g., eugenol and *trans*-cinnamaldehyde) (Tables 2 and 3). In milk, the increased EOC concentration in the milk serum prepared by the EPM (Fig. 3) enables the enhanced reduction of bacteria, and the quicker mixing of EOCs in the EPM than in the ETEM corresponds to greater log reductions in shorter times (Tables 4 to 6). The antimicrobial activity again can be interpreted by comparing the EOC concentrations in the milk serum at 0 and 24 h (Fig. 3) with the corresponding MICs/MBCs (Table 3).

In terms of specific EOCs, thymol and carvacrol generally have better antilisterial activity than eugenol and *trans*-cinnamaldehyde (Tables 3 to 6). This trend is consistent with the structure characteristics observed for EOCs, showing antimicrobial activity following the order phenols > aldehydes > ketones > alcohols > esters > hydrocarbons (38). *trans*-Cinnamaldehyde is more polar than the other three EOCs in the present study (greatest water solubility) (Table 2) and therefore may be less effective in affecting the structure of the cytoplasmic membrane (33). In milk, the aldehyde group of *trans*-cinnamaldehyde may allow stronger binding with proteins than the hydroxyl group of thymol, carvacrol, and eugenol (Table 1). Binding between hydrophobic compounds and globulin proteins, like β-lactoglobulin, can be a long process (5), and this may have caused the lower concentration of *trans*-cinnamaldehyde in the serum after mixing with skim milk for 24 h (Fig. 3D). The difference in binding properties may have caused the incomplete inhibition of the growth of *L. monocytogenes* in full-fat milk by *trans*-cinnamaldehyde (Table 6), although its concentration in the milk serum is higher than the MBC (Fig. 3 versus Table 3). Furthermore, the log reduction at the 0-h time point was different when 2.5 g/liter thymol and carvacrol were applied in skim milk using the ETEM (Table 4). Carvacrol is an isomer of thymol (39) and has similar MICs and MBCs (Table 3). Unlike the crystal form of thymol, carvacrol is present as a liquid at room temperature, which allows quick mixing in the ETEM and likely causes the difference between the 2.5-g/liter thymol and carvacrol treatments. The quicker mixing of 2.5 g/liter carvacrol in skim milk is also consistent with the same log reductions at 0 h using both the ETEM and EPM, contrasted with the significant difference for 2.5 g/liter thymol prepared by the two methods (Table 4).

Although the qualitative correlation between the antimicrobial activities of EOCs and their concentrations in the milk serum can be made by referencing to the MIC/MBC, the quantitative correlation of treatments prepared by the ETEM and EPM does not appear to be straightforward. Growth of *L. monocytogenes* in milk with low concentrations of ethanol after 24 h (Tables 4 to 6) makes it impossible to correct data from the EPM to eliminate the effect of ethanol. Because ethanol is generally not present in nonfermented foods, the EPM should be avoided if possible to assess realistic antimicrobial properties of EOCs expected in foods. From this perspective, mechanical methods of incorporating EOCs, such as the ETEM evaluated in the present study, should be used to achieve solubility of EOCs and to evaluate delivery systems intended to improve the distribution and dissolution of EOCs. Conversely, the EPM offers convenience in sample preparation and can be used to enhance antimicrobial activity. When the EPM is used to prepare controls in studying delivery systems of antimicrobials, the increased antimicrobial activity by low concentrations of ethanol may result in inappropriate conclusions about the

potential of delivery systems to improve the activity of EOCs in food matrices. Methods to address such scenarios require future work.

Conclusions. Overall, the solubility and antimicrobial characteristics of EOCs were affected by the preparation methods. The solubility of EOCs was increased by low concentrations of ethanol, and the increased solubility lowered the MICs and MBCs of EOCs with solubility comparable to the MBC (eugenol and *trans*-cinnamaldehyde). In milk serum, the EOC concentration was reduced to a greater extent by a higher fat level and was increased by low concentrations of ethanol resulting from the EPM. The EPM enabled faster distribution of EOCs to a higher level, which corresponded to the enhanced log reductions of *L. monocytogenes* after short (0-h) and long (24-h) exposures. The EOC concentration quantified in the milk serum was correlated with log reductions of *L. monocytogenes* by comparison with the MIC and MBC determined in TSB. Generally, an EOC concentration in milk serum higher than the MBC corresponded to complete inactivation of *L. monocytogenes*, while one between the MIC and the MBC agreed with partial reductions of the bacteria. Since the EPM increases antimicrobial activity of EOCs in milk, the ETEM is a more realistic representation of the antimicrobial effectiveness expected in real food systems free of alcohol. The EPM offers convenience in sample preparation in laboratories, but the calibration of antimicrobial activity in foods due to low concentrations of alcohol remains a research question.

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